Remarks

This is in response to the Official Action of February 14, 2003. The points raised therein are addressed below in the order originally set forth.

Claims 1-10 stand rejected under the first paragraph of 35 USC 112 as containing subject matter which was not described in the specification in such a way as to reasonably convey tone one skilled in the relevant art that the inventor had possession of the claimed invention at the time the application was filed. For the reasons set forth below, this rejection is respectfully traversed.

It is alleged on page 2 of the official action that "there is no disclosure regarding the characteristics of the transgenic mouse." Applicants respectfully disagree. A "human pattern of expression" for mice of the instant invention is amply defined in the specification at page 3, lines 16-26. The manner for determining a human pattern of expression is further discussed in the Examples at page 10, lines 21-24 (testing of peritoneal macrophages).

In *Pfaff v. Wells Electronics*, 48 USPQ2d 1641 (1998), the United States Supreme Court held that an invention is "ready for patenting" when "the inventor has prepared drawings or other descriptions of the invention that were sufficiently specific to enable a person skilled in the art to practice the invention." *Id.* at 1647. Such is the case here: transgenic mice produced as described in the instant application possess the human pattern of expression recited in the instant application (See **EXHIBIT A** submitted concurrently herewith, particularly as it deals with nitrite measured from stimulated peritoneal macrophages, which applicants will be pleased to submit in the form of a Rule 132 Declaration if the Examiner so desires).

Claims 1-10 stand rejected under 35 USC 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. This rejection is respectfully traversed. It is respectfully submitted that the enablement requirement is satisfied in the instant case for the same reasons as the possession requirement described above. Further, it is noted that the human NOS2 gene is known (see the specification at page 3, lines 5-7), mice containing an

inactive endogeneous mouse inducible nitric oxide synthase gene are commercially available (see the specification at page 3, lines 8-10), and methods of producing transgenic mice from such starting materials are well known (see, e.g., the specification at page 3, lines 11-15). In addition, with respect to use of the mouse as a screening tool, it is respectfully submitted that, with a transgenic mouse in hand, screening various compounds for particular activities can be carried out in a routine manner, as has been done in mouse testing for many decades. Accordingly, it is respectfully submitted that this rejection should be withdrawn.

Claims 1-10 stand rejected as indefinite under 35 USC 112 in item 7 of the Official Action, various points in the claims being noted by the Examiner. Applicants respectfully disagree, it being respectfully submitted that the meaning of the claims is rendered clear by the specification. Nevertheless, to simplify and narrow the issues, the claims have been amended consistent with the specification to obviate the concerns raised by the examiner. All of these amendments are consistent with the meaning of the claims established by the specification and hence it is respectfully submitted that such amendments are not narrowing amendments. It is respectfully submitted that these rejections may be withdrawn.

Claims 1-10 stand rejected as obvious under 35 USC 103 over Woi et al. in view of Chartrain et al. and Cameron. This rejection is respectfully traversed. Woi et al. is said to teach iNOS knockout mice, Chartrain is sead to teach the human iNOS gene, Cameron is said to review the state of the art of making transgenic mice. It is further said that "an artisan of skill would have been motivated to make such a transgenic mouse because as taught by Chartrain et al., the human gene needed to be characterized and the transgenic mouse of Wei et al. would have allowed to define the function of the human iNOS gene in a mouse which lacked its endogeneous iNOS gene." Applicant respectfully disagrees. Chartrain et al. specifically state "The human iNOS gene's putative promoter region was strikingly similar to the murine iNOS promoter, suggesting at least a partial conservation of regulatory function. However, due to the apparent differences between human and murine iNOS expression (*e.g.*, macrophage expression), detailed functional analysis of the human iNOS promoter is necessary before the complexities of human iNOS gene regulation can be fully understood." Clearly what is suggested by Chartrain et al. is a deletion analysis of the human iNOS

promoter, and not insertion of the human iNOS gene and regulatory elements into a transgenic mouse. Hence, it is respectfully submitted that the cited references do not provide a motivation to combine one another as required for a rejection under 35 USC 103 (*see, e.g.*, *In re Sang-Su Lee*, 61 USPQ2d 1430 (Fed. Cir. 2002), and it is respectfully submitted that this rejection should be withdrawn.

It is submitted that this application is in condition for allowance, which action is respectfully requested.

Respectfully submitted,

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Enclosure: **Exhibit A**

Nitric Oxide Production in a Transgenic Mouse Displays Human Patterns of Nitric Oxide Production

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EXHIBIT A
09/744,133

Abstract:

Chronic inflammation is a common component of multiple human diseases in the central nervous system and periphery. To understand how human inducible nitric oxide synthase (iNOS) contributes to chronic inflammation seen in chronic neurodegenerative pathophysiologies such as Alzheimer's disease, we constructed a transgenic mouse model that expresses only human iNOS. Using RT-PCR and Western blotting, these mice were shown to express only human iNOS RNA and protein in stimulated whole brain and peritoneal macrophages. Since microglia are the brain-specific macrophage, we studied NO responses in peritoneal macrophages as a model for brain microglia. Specifically, we measured nitrite production as an indirect measure of NO release, and iNOS catalytic activity, a direct measure of iNOS enzymatic function. Nitrite measured from stimulated peritoneal macrophages from adult (4-5 months) and aged (20-24 months) mice displayed a human pattern of NO production. These data show that this mouse is an excellent tool to study human-specific patterns of nitrosative stress that contribute to neuroinflammation in a mouse model system.

Introduction:

Inducible nitric oxide synthase (iNOS) is a member of the family of nitric oxide synthases that produce nitric oxide (NO) from the enzymatic conversion of L-arginine to L-citrulline. The iNOS protein controls several aspects of inflammation, including mediating the microbial and tumoricidal response to infection. It regulates a high output, highly regulated NO output that can be beneficial (antiinflammatory, antiapoptic) or deleterious ( ), depending on the cellular microenvironment. Multiple diseases are linked to iNOS physiology and pathophysiology, including arthritis, inflammatory bowel disease, malaria, and, more recently, neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).

The iNOS protein is encoded by the NOS2 gene located on chromosomes in mouse and humans, respectively. Although the function of iNOS is similar in both mouse and humans, NOS2 gene regulation displays remarkable species specificity. The most striking differences are located in the NOS2 promoter for each species where the number, type, and location of transcription factor binding sites differ. The regulatory sequences that are necessary and sufficient for mouse NOS2 transcription are located -1.6 kB upstream of the transcription start site and contains primarily NF-kB, LPS, and interferon gamma (IRE) immune response elements ( ). In contrast, the NOS2 promoter

Methods:

Transgenic Mouse Construction-

The human NOS2 gene sequence was used to obtain several PAC clones of human genomic DNA containing all 65 kb of the human NOS2 gene, including 15 kb of gene regulatory sequences flanking 40 kb of exons and introns (insert ref). Each clone was subjected to multiple PCR reactions, sequenced, and restriction mapped to confirm that they were positive for and correctly matched the published promoter/exon-1 region to exon 27 region of the human iNOS gene. A single PAC clone meeting the appropriate criteria was used to generate a transgenic mouse. The purified PAC clone was purified and linearized with Not 1. Not 1 does not cleave the human NOS2 gene (ref). Linearized DNA was injected into the pronucleus of a fertilized mouse egg at the Duke University Transgenic Mouse Facility using standard methods. Four F0 founders were identified and designated A-D. Genomic DNA was extracted from the tail of each founder and subjected to PCR using primers from the human NOS2 promoter/exon 1 region (forward primer= 5'-CCTTTCCCTTCCAAAACCTC-3'; reverse primer= 5'-TCACCCAACCCACCTCTTTC-3'.) Genomic DNA from founders was also subjected to Southern blotting. DNA was digested with Hind III and hybridized with a pIN-2 probe, spanning positions +2401 to +4203 of the human iNOS cDNA and corresponding to exons 18-27 of the human iNOS gene. When hybridized to transgenic mouse genomic DNA, presence of the human iNOS gene is indicated by multiple bands of 3.6 kb, 4 kb, 4.5 kb, 6 kb, 8 kb, 9.5 kb, and 12 kb, while the presence of mouse iNOS gives one 7.6 kb band. Transgenic mice contained both human and mouse specific bands as described. A second complementary Southern blot was employed utilizing a BamHI digest to produce a 14 kb band in non-transgenics and 14 kb and 2 kb bands in transgenic animals using a probe corresponding to human iNOS exon 18. F_0 mice containing the human NOS2 gene were mated to wild-type (WT) C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) to produce a hemizygous F1 line used for further propagation. Lines A-C were selected for further propagation and study.

Preparation of a Humanized NOS2 mouse-
F1 progeny from lines A-C were then mated to iNOS knockout mice in two subsequent breedings (iNOS-KO) (Jackson Labs) to generate a hemizygous humanized iNOS mouse, i.e. a mouse that expresses only human iNOS gene products and no mouse iNOS gene products; these mice were designated: hNOS2Tg/mNOS2KO. The presence of the human iNOS gene was confirmed using promoter/exon 1 primer pairs described above, producing an expected 477 bp product or exon 27 primer pairs (get primer sequence) to produce a 600 bp (???) product. Animals from the B line were the most robust breeders and were used for the duration of all further studies.

Cell and Tissue Collection-
Whole mouse brains and peritoneal macrophages were collected from mice injected intraperitoneally (i.p.) with $NaIO_4$ (Sigma, St. Louis, MO) or vehicle (sterile 1X PBS). Briefly, iNOS induction was elicited by two intraperitoneal (i.p.) injections of $NaIO_4$ as previously described (Weinberg, 1983 #29; Weinberg, 1981 #514). Mice received 1 ml 5 mM NaIO4 followed on Day 0, followed a second injection on Day 3, and were sacrificed on Day 4 of the experiment. Mice were rendered unconscious using carbon dioxide and subsequently decapitated. Whole brain tissue from female and male hiNOSTg/miNOSKO mice was collected for RNA and protein analysis; brains were separated into two hemispheres, snap frozen in liquid nitrogen, and stored at -80°C. Peritoneal macrophages were collected in 3-5 ml of ice cold 10 units/ml heparin in 1X PBS and pooled together depending on genotype and injection paradigm. Macrophages were washed using distilled water to remove red blood cells followed by addition of 2X PBS to restore isotonicity. Cells were centrifuged at 1000 rpm for 10 min; supernatant was aspirated followed by resuspension of the pellet in 15-20 ml macrophage media: 1% L-glutamine, 1% penicillin/streptomycin, 1% HEPES, 10% fetal calf serum in high glucose DMEM (all purchased from Gibco BRL, Rockville, MD.)

Treatment of Peritoneal Macrophages-
Peritoneal macrophages were plated in 96 well plates at a density of $1X10^5$ cells/well in 200 μl of macrophage media and incubated at 37°C in 95% O_2/5% CO_2. To induce iNOS and NO production, cells were treated with various inflammatory stimulators including: mouse recombinant interferon γ, polyinosinic:polycytidylic acid (PIC), lipopolysaccharide (LPS): E.coli serotype 0111:B4 (all purchased from Sigma), human recombinant interferonγ, mouse recombinant IL-1β, human recombinant IL-1β, human TNFα (R&D Systems, city, state). Cells were treated for approximately 5 days (72-120 h) in 200 ul serum free macrophage media.

RNA Preparation and RT-PCR-
RNA was isolated from peritoneal macrophages and brain tissue using TRIzol (Gibco BRL, Rockville, MD), according to manufacturer's instructions. RNA concentrations were measured using a Beckman spectrophotometer (city, state). For RT-PCR, first strand DNA synthesis was performed using Qiagen's Sensiscript (for peritoneal macrophage RNA) or Omniscript (for whole brain RNA) Kit (city, state) according to manufacturer's directions. A 1 μl aliquot of product was used in the second strand standard PCR reaction using human iNOS specific primers exon27for 5'- -3' and exon27rev 5'- -3' with the following cycles: 94 C for 5', followed by denaturing – 1' at 94 C, annealing – sec at 60 C, extension for 1' at 72 C, for 30 cycles followed

by a final extension step for 7 min at 72 C. GAPDH primers, GAPDHF 5'- 3', and GAPDHR 5'- 3', were used as internal controls using PCR cycles previously described except that a 55 C annealing temperature was used for 25 cycles. Gels were stained using SYBR green (Molecular Probes, city, state) and visualized using a KODAK Image Station 440CCF System (Eastman Kodak, Rochester NY). Kodak 1D Image Analysis Software (Eastman Kodak, Rochester NY) was used for gel image analysis.

Western Blotting-
Peritoneal macrophages and whole brain tissue were sonicated in 1 ml or 2 ml, respectively, of protease inhibitor solution (Sigma). Protein concentrations were measured using BCA (Pierce,) or BioRad Protein Reagent (BioRad,) with BSA (ug/ml) as standard according to manufacturer's directions. 20-40 ug protein was loaded onto a 10%-20% Tricine gel (Invitrogen) in 2X Laemmli sample buffer (Novex?) and run at 100 mV for 2 hrs. Proteins were then transferred onto PVDF membrane (Millipore,) for 2hrs, blocked into Blotto (5% dry milk in TBST) at room temperature for 1 hr, followed by incubation in human iNOS primary antibody at 1:1000 (R&D) or mouse/human iNOS antibody at 1:1000 (Transduction Laboratories,), or GAPDH as a loading control (1:10000, Sigma?) . Blots were washed three times in TBST followed by 1 hr incubation with the appropriate secondary antibody, subjected to 3 washes in TBST, followed by visualization using ECL-Plus chemiluminescence (Amersham,), and analyzed using a Kodak Imager and its accompanying software.

Measurement of nitrite and total protein-
Nitrite levels were determined by combining 100 µl media with 100 µl Griess reagent and incubated at room temperature for 20 minutes. Griess reagent was prepared by mixing equal volumes of reagent #1 (1% sulfanilic acid/5% phosphoric acid) and reagent #2 (0.1% napthylethylenediamine) (all purchased from Sigma). Additional nitrite levels were also measured by injecting 50 µl media sample into a Sievers 280 NOA analyzer (Boulder, CO). Total protein (µg/well) was measured using the BCA method (Pierce, Rockford, IL) according to manufacturer's instructions with BSA (µg/ml) as standard. Both Griess and BCA values were measured using a Molecular Devices Theromax Microplate Reader (Menlo Park,CA) at OD_{562}. Nitrite levels were expressed as µM NO_2-/µg protein. Cell viability was measured using a standard MTT assay. Briefly, cells were incubated with 25 µl of 5 mg/ml MTT (Sigma) prepared in 1X PBS at 37°C, 95%O_2/5% CO_2 for 4 h, followed by addition of 100 µl lysis buffer overnight to solubilize the stable mitochondrial purple foramazoan product, and read at OD_{562}.

Measurement of iNOS Activity-
Nitric oxide synthase in elicited, immune-stimulated peritoneal macrophages was performed measuring the conversion of 14C-L-arginine to 14C-L-citrulline. Cell lysates were obtained by removing the treatment media and collected using a cell scraper into 1.5ml macrophage NOS activity buffer (ultrapure water (Gibco), 1X protease inhibitors (Sigma), 100uM PMSF). Cells were pelleted for 10 minutes at 1000rpm, 4 C; the pellet was resuspended and lysed using vigorous pipetting in 30 – 40 ul macrophage NOS activity buffer. The solution was centrifugated at 14,000rpm for 25 minutes at 4 C. Total protein content of the cell lysate was measured using the BioRad Protein Reagent (BioRad,).
Activity reactions were performed in triplicate using 10ul protein lysate in a 50ul total reaction volume ( ). Reactions were carried out for 60 minutes at 37 C. Each reaction was

terminated by eluting the reaction mixture over a Dowex 50WX8 ion exchange resin (converted to Na+ form) and washing in 3 volumes of stop buffer ( ). Total dpm of 14C-L citrulline were counted a Beckman Tri-Carb scintillation counter ( ). All values were expressed as pMol/mg protein/hour.

Statistical Analysis-
Nitrite (μM)/μg protein is expressed as mean ± SEM. Each data point represents the mean of at least six wells. Data were analyzed using two-tailed paired or unpaired student's t-test and one-way or repeated measures ANOVA and Tukey's post test as appropriate with Graphpad Prism and Instat software packages (San Diego, CA). Values of $p < 0.05$ (*) were considered significant, $p < 0.01$ (**) very significant, and $p < 0.001$ (***) extremely significant.

<u>Results:</u>
Expression of hiNOS mRNA and protein-
Total RNA and protein were collected from whole brain tissue and murine peritoneal macrophages from male and female human iNOS transgenic mice to examine tissue specific expression levels of human iNOS in stimulated (5 mM $NaIO_4$, i.p.) and unstimulated cells. Figure 1A illustrates that a 2XX bp human iNOS fragment is expressed only in brains of male (lanes x – x) and female (x – x) human iNOS transgenic mice, while no PCR fragment was detected in wildtype C57Bl/6 mice. GAPDH fragment intensity was compared to human iNOS fragment intensity in each individual lane and no significant differences were detected in mRNA levels between males and females or between 5mM $NaIO_4$ or 10% peptone stimulation (data not shown). Figure 1B illustrates that a 130 kD band for human iNOS was present in stimulated whole brain tissue from both male and female mice using Western blotting, with GAPDH used as a loading control. Human iNOS fragment intensities for each sample were compared to GAPDH and found not to be significantly different between males or females (data not shown). These results demonstrate that only human iNOS mRNA and protein are expressed in this transgenic mouse.

Nitrite production in human NOS2 transgenic mice: mouse versus human-
One goal of the study was to determine if the difference in levels of nitrite production between rodents and humans was due to the presence the structure of the human or mouse genes, or due to the unique cell biology of human and mouse cell. These hypotheses were tested by measuring nitrite production using both human and mouse recombinant cytokines in combination with typical murine and human patterns of immune stimulation. Figure 2 shows nitrite production in 4-5 month old male peritoneal macrophages stimulated for 5 days using typical murine patterns of immune stimulation that included: Poly I:C (PIC), a viral mimetic; lipopolysaccharide (LPS), a bacterial endotoxin; and murine recombinant interferon gamma (mIFNγ). Nitrite was measured using the Griess reaction and chemiluminescence and found to be similar to published nitrite levels from stimulated human monocytes (insert ref). Nitrite levels were detectable at the only at the nmol level after 5 days, a result in sharp contrast to normal murine nitrite levels that are easily detectable at the umol level after 24 – 48 hrs. Stimulation using mIFNγ (2.5ng/ml) and LPS (500ng/ml) produced the highest level of nitrite output at 800 nmol/ug. All nitrite production was significantly higher than that from untreated peritoneal macrophages ($p<0.001$, ANOVA).

Nitrite production from 4-5 month old male peritoneal macrophages stimulated using typical human patterns of immune stimulation is shown in Figure 3. Nitrite production in peritoneal macrophages was lower in these cells than the cells treated with mouse immune stimulation (Figure 2); the highest level of nitrite production was at 300 nmol/ug versus 800 nmol/μg for murine stimulation. Murine IL-1β (0.2 ng/ml) and human IL-1b (0.2 and 2 ng/ml) were capable of producing a nitrite response alone which is normally not detectable in normal wildtype mouse peritoneal macrophages. Combinations of murine and human IL-1b with both mouse and human IFNg were also capable of synergistically producing an immune response that was significantly higher than treatment with IL-1b alone ($p<0.$ – insert statistic from raw data). Immune stimulation was also detected using human TNFa (10ng/ml) that were significantly higher than unstimulated cells. However, immune stimulation of transgenic macrophages with a combination of cytokines including IFNg, IL-1B, and TNFa produced a nitrite response that was insignificant compared to unstimulated macrophages.

Nitrite Production in Aged NOS2 Transgenic Mouse:

Human iNOS transgenic mice were aged to 20-24 months and nitrite production was measured to assess differences in the human iNOS inflammatory response during advanced age.

iNOS Activity in NOS2 Transgenic Mouse

Discussion

Multiple studies using human mononuclear phagocytes have reported nitrite production only by using a combination of various human cytokines (insert ref), while others have reported

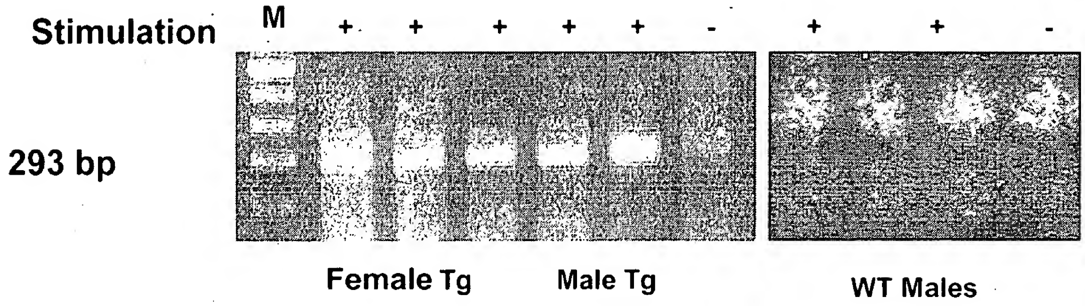
Figure Legends

Acknowledgements: We wish to acknowledge Michael H. Herbstreith for excellent technical support for maintenance of these mouse strains and Dr. Carol A. Colton for helpful comments and insightful discussions.

References:

Figure 1: Human iNOS Transgenic Mice Express Only Human iNOS

A. RTPCR



B. Western

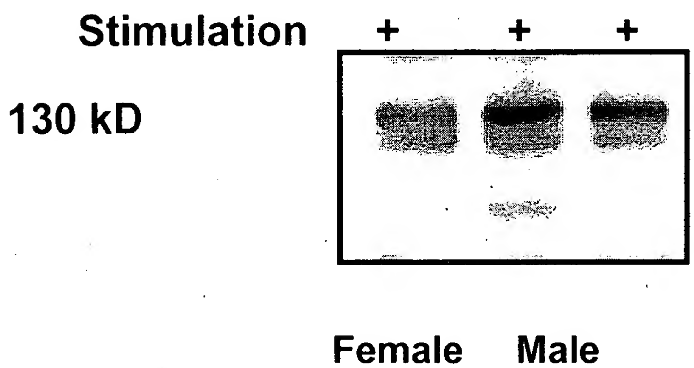
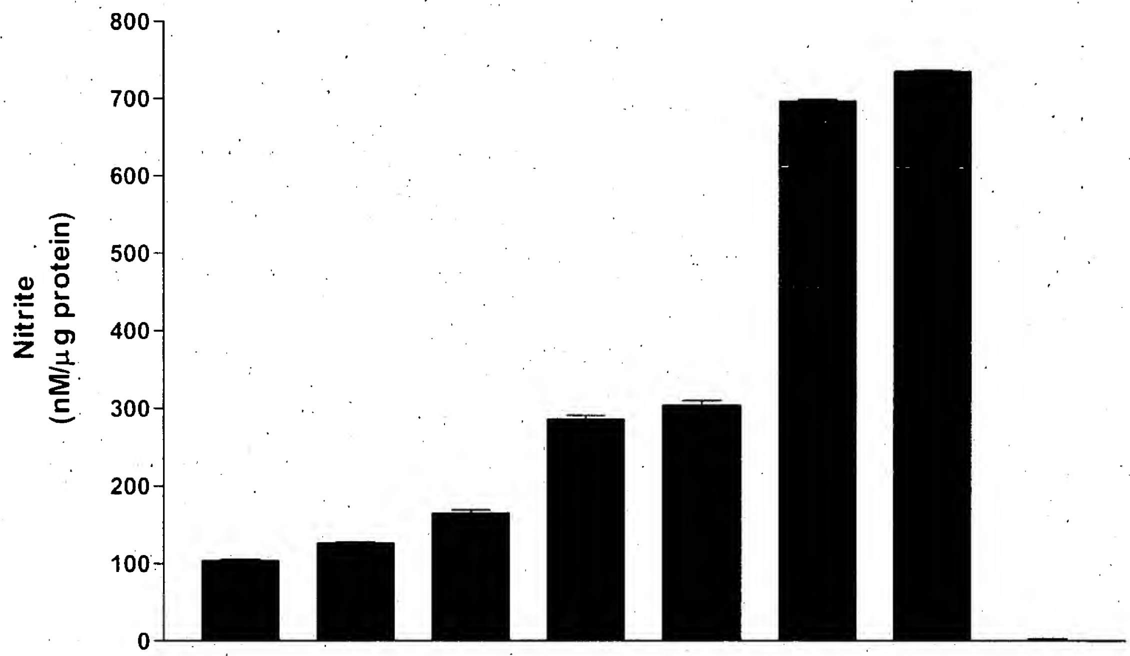
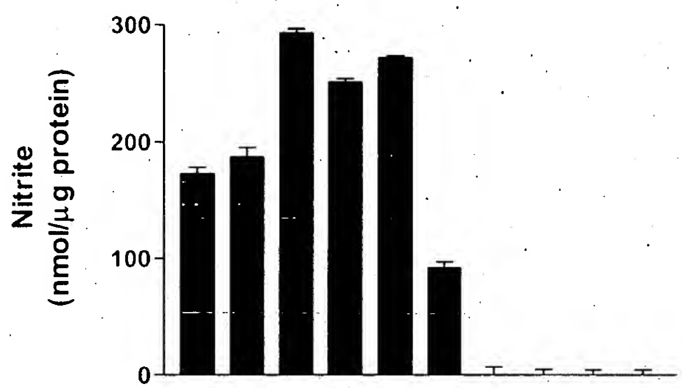


Figure 2: Nitrite Production in an Adult Human iNOS Transgenic Mouse Stimulated Using Typical Mouse-Specific Stimulators



mIFNγ (10u/ml)	+	-	-	+	+	+	+	-
PIC (µg/ml)	-	100	-	50	100	-	-	-
LPS (ng/ml)	-	500	-	-	-	100	500	-

Figure 3: Nitrite Production in an Adult Human iNOS Transgenic Mouse Using Human-Specific Immune Stimulation



mIFNγ (10u/ml)	-	-	-	-	+	-	-	-	-	-
hIFNγ (7ng/ml)	-	-	-	+	-	-	+	-	+	-
mIL-1β (ng/ml)	0.2	-	-	-	2	-	-	-	-	-
hIL-1β (ng/ml)	-	0.2	2	2	-	-	-	2	2	-
hTNFα (10ng/ml)	-	-	-	-	-	+	+	+	+	-

Figure 4: Nitrite Production in an Aged Human iNOS Transgenic Mouse Using Both Murine and Human Specific Immune Stimulation

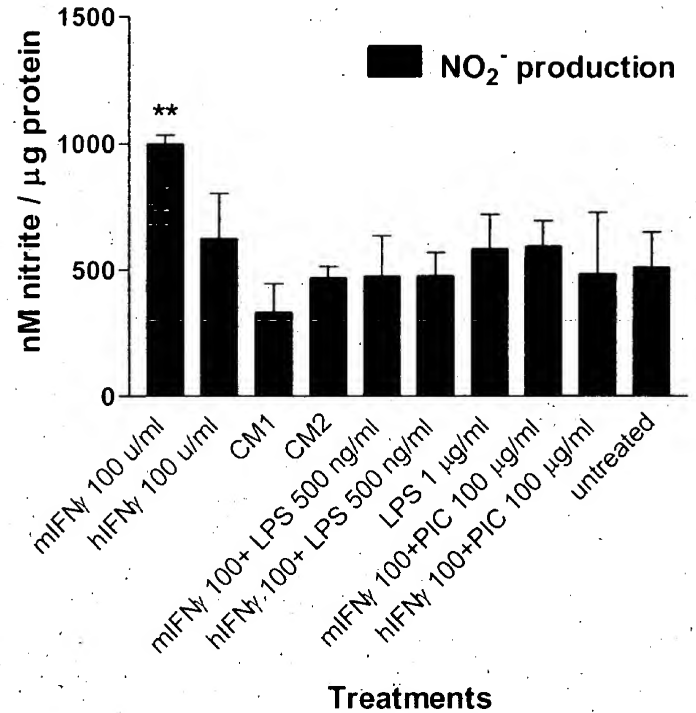


Figure 5: iNOS Activity in Peritoneal Macrophages from the human NOS2 transgenic mouse